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(54) Title: CLONING OF THE PROLYL-DIPEPTIDYL-PEPTIDASE FROM <i>ASPERGILLUS ORYZAE</i> (57) Abstract The invention has for object the new recombinant prolyl-dipeptidyl-peptidase enzyme (DPP IV) from <i>Aspergillus oryzae</i> comprising the amino-acid sequence from amino acid 1 to amino acid 755 of SEQ ID NO:2 or functional derivatives thereof, and providing a high level of hydrolysing specificity towards proteins and peptides starting with X-Pro- thus liberating dipeptides of X-Pro type, wherein X is any amino acid. The invention also provides a DNA molecule encoding the enzyme according to the invention, cells expressing the enzyme according to the invention by recombinant technology, an <i>Aspergillus</i> naturally providing a prolyl-dipeptidyl-peptidase activity which has integrated multiple copies of the <i>Aspergillus</i> native promoter which naturally directs the expression of the gene encoding the prolyl-dipeptidyl-peptidase activity, <i>Aspergillus</i> naturally providing a prolyl-dipeptidyl-peptidase activity which is manipulated genetically so that the <i>dppIV</i> gene is inactivated. The invention provides a method for producing the enzyme according to the invention, comprising cultivating the cells of the invention in a suitable growth medium under conditions that the cells express the enzyme, and optionally isolating the enzyme in the form of a concentrate. The invention provides the use of the enzyme or the cells of the invention to hydrolyse protein containing materials. The invention provides the use of an enzyme and/or a cell providing a prolyl-dipeptidyl-peptidase activity, in combination with at least an enzyme providing a prolylase to hydrolyse protein containing materials. In a last further aspect, the invention provides a food product comprising a protein hydrolysate obtainable by fermentation with at least a microorganism providing a prolyl-dipeptidyl-peptidase activity higher than 50 mU per ml when grown in a minimal medium containing 1 % (w/v) of wheat gluten.		

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Cloning of the prolyl-dipeptidyl-peptidase from *Aspergillus oryzae*

The present invention relates to a new recombinant prolyl-dipeptidyl-peptidase from *Aspergillus oryzae*, a gene encoding this enzyme, recombinant cells
5 expressing this enzyme, and methods for hydrolysing protein containing materials.

State of the art

Hydrolysed proteins, which are widely used in the food industry, may be prepared
10 by hydrolysis of protein material with acid, alkali or enzymes. However, on the one hand, acid or alkaline hydrolysis can destroy the essential amino acids produced during hydrolysis thus reducing the nutritional value, whereas enzymatic hydrolysis rarely goes to completion so that the hydrolysed protein contains substantial amounts of peptides.

15 The filamentous ascomycete *Aspergillus oryzae* is known to secrete a large variety of amylases, proteinases and peptidases, the action of which are essential for the efficient solubilisation and hydrolysis of raw materials (see WO94/25580). Various methods have been used *Aspergillus oryzae* for the preparation of food
20 products, especially methods involving the use of a koji culture.

EP417481 (Nestlé) thus describes a process for the production of a fermented soya sauce, in which a koji is prepared by mixing an *Aspergillus oryzae* koji culture with a mixture of cooked soya and roasted wheat, the koji is then hydrolysed in
25 aqueous suspension for 3 to 8 hours at 45°C to 60°C with the enzymes produced during fermentation of the *Aspergillus oryzae* koji culture, a moromi is further prepared by adding sodium chloride to the hydrolysed koji suspension, the moromi is left to ferment and is then pressed and the liquor obtained is pasteurized and clarified.

30 EP429760 (Nestlé) describes a process for the production of a flavouring agent in which an aqueous suspension of a protein-rich material is prepared, the proteins are solubilized by hydrolysis of the suspension with a protease at pH6.0 to 11.0, the suspension is heat-treated at pH 4.6 to 6.5, and the suspension is ripened with
35 enzymes of a koji culture fermented by *Aspergillus oryzae*.

Likewise, EP96201923.8 (Nestlé) describes a process for the production of a meat flavour, in which a mixture containing a vegetal proteinaceous source and a vegetale carbohydrates containing source is prepared, said mixture having initially at least 45% dry matter, the mixture is inoculated with a koji culture fermented by
5 *Aspergillus oryzae* and by one or more another species of microorganisms involved in the traditional fermentation of meat, and the mixture is incubated until meat flavours are formed.

Depending on the nature of the protein and the enzymes used for proteolysis, the
10 peptides formed can however have extremely bitter tastes and are thus organoleptically undesirable. There is hence a need for methods of hydrolysing proteins leading to high degree of protein hydrolysis and to hydrolysates with excellent organoleptic properties.

15 In addition, in protein rich materials subjected to enzymatic hydrolysis, a high level of glutaminase is required to convert glutamine into glutamic acid which is an important natural taste enhancer (see WO95/31114). Biochemical analysis of residual peptides in cereals hydrolysed by *Aspergillus oryzae*, i.e. wheat gluten, shows however that a considerable amount of glutamine remains sequestered in
20 proline containing peptides (Adler-Nissen, *In: Enzymatic hydrolysis of food proteins*. Elsevier Applied Sciences Publishers LTD, p120, 1986). There is hence a need for methods of hydrolysing proteins leading to liberation of high amount of glutamine.

25 Among the different proteases known from koji molds, two neutral endopeptidase (Nakadai *et al.*, Agric. Biol. Chem., 37, 2695-2708, 1973), an alkaline endopeptidase (Nakadai *et al.*, Agric. Biol. Chem., 37, 2685-2694, 1973), an aspartic protease (Tsujita *et al.*, Biochem. Biophys Acta, 445, 194-204, 1976), several aminopeptidases (Ozawa *et al.*, Agric. Biol. Chem., 37, 1285-1293, 1973),
30 several carboxypeptidases (Nakadai *et al.*, Agric. Biol. Chem., 37, 1237-1251, 1970) have been identified and purified.

More recently a prolyl-dipeptidyl-peptidase activity has been detected in
35 *Aspergillus oryzae*, which is an enzyme providing a high level of hydrolysing specificity towards proteins and peptides starting with X-Pro- thus liberating dipeptides of X-Pro type, wherein X is any amino-acid (Tachi *et al.*,

Phytochemistry, 31, 3707-3709, 1992).

Summary of the invention

5 The present invention has for object the new recombinant prolyl-dipeptidyl-peptidase (DPP IV) from *Aspergillus oryzae* comprising the amino-acid sequence from amino acid 1 to amino acid 755 of SEQ ID NO:2 or functional derivatives thereof.

10 In a second aspect, the invention also provides a DNA molecule encoding the enzyme according to the invention.

In a third aspect, the invention provides a cell expressing the enzyme according to the invention by recombinant technology.

15

In a fourth aspect, the invention provides an *Aspergillus* naturally providing a prolyl-dipeptidyl-peptidase activity which has integrated multiple copies of the *Aspergillus* native promoter which naturally directs the expression of the gene encoding the prolyl-dipeptidyl-peptidase activity.

20

In a fifth aspect, the invention provides an *Aspergillus* naturally providing a prolyl-dipeptidyl-peptidase activity which is manipulated genetically so that the *dppIV* gene is inactivated.

25 In a sixth aspect, the invention provides a method for producing the enzyme according to the invention, comprising cultivating the cells of the invention in a suitable growth medium under conditions that the cells express the enzyme, and optionally isolating the enzyme in the form of a concentrate.

30 In a seventh aspect, the invention provides the use of the enzyme or the cells of the invention to hydrolyse protein containing materials.

In another aspect, the invention provides the use of an enzyme and/or a cell providing a prolyl-dipeptidyl-peptidase activity, in combination with at least an
35 enzyme providing a prolidase to hydrolyse protein containing materials.

In a last further aspect, the invention provides a food product comprising a protein hydrolysate obtainable by fermentation with at least a microorganism providing a prolyl-dipeptidyl-peptidase activity higher than 50 mU per ml when grown in a minimal medium containing 1 % (w/v) of wheat gluten

5

Detailed description of the invention

Within the following description, the percentages are given by weight except where otherwise stated, and the amino acid or nucleotide sequences referred as "SEQ ID NO:" are always presented in the sequence listing hereafter.

10

Likewise, the expression "functional derivative of an enzyme" includes all amino acid sequences which differ by substitution, deletion, addition of some amino acids, for instance 1-20 amino acids, but which keep their original activities or functions. The selection of a functional derivative is considered to be obvious to one skilled in the art, since one may easily creates variants of the DPP IV (having the amino acid sequence SEQ ID NO:2) by slightly adapting methods known to one skilled in the art, for instance the methods described by Adams et al. (EP402450; Genencor), by Dunn et al. (Protein Engineering, 2, 283-291, 1988), by Greener et al. (Strategies, 7, 32-34, 1994), and/or by Deng et al. (Anal. Biochem, 200, 81, 1992).

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In particular, a protein may be generally considered as a derivative to another protein, if its sequence is at least 80% identical to the protein, preferably at least 90%, in particular 95%. In the context of the present disclosure, the identity is determined by the ratio between the number of amino acids of a derivative sequence which are identical to those of the DPP IV having the amino acid sequence SEQ ID NO:2 (mature sequence 1-755), and the total number of or amino acids of the said derivative sequence.

25

30

In addition, the term "koji" designates the product of the fermentation with a koji mold culture of a mixture of a source of proteins and a source of carbohydrates, especially of a mixture of a leguminous plant or of a cooked oleaginous plant and of a cooked or roasted cereal source, for example of a mixture of soya or cooked beans and of cooked or roasted wheat or rice.

35

The present invention thus concerns the new prolyl-dipeptidyl-peptidase enzyme originating from *Aspergillus oryzae* which comprises the amino-acid sequence from amino acid 1 to 755 of SEQ ID NO:2 or functional derivatives thereof. This enzyme may be operably fused to a leader peptide facilitating its secretion in a host where the enzyme is expressed, for example the *Aspergillus oryzae* leader peptide having the amino-acid sequence from amino acid -16 to -1 of SEQ ID NO:2 or functional derivatives thereof.

A *dppIV* gene encoding the DPP IV according to the invention may at least comprise the coding parts of the nucleotide sequence SEQ ID NO:1, or functional derivatives thereof due to the degeneracy of the genetic code. This sequence is in fact interrupted by a non-coding sequence, called intron, that is spliced during *in-vivo* transcription (exon I at 1836-1841 bp; exon II at 1925-1924 bp; intron at 1842-1924 bp).

A *dppIV* gene may be obtained in substantially purified form by using the method described within the following examples from any strain of *Aspergillus oryzae*. Alternatively, a *dppIV* gene may be (1) detected also from other genera or species of microorganisms by use of DNA probes derived from the nucleotide sequence SEQ ID NO:1 in a stringent hybridization assay, and (2) recovered by the well known Reverse-PCR method by use of suitable primers, for example primers SEQ ID NO:8 and 9. In a further aspect, a *dppIV* gene may also be *in-vitro* synthesized and then multiplied by using the polymerase chain reaction, for instance.

The DNA molecule according to the invention at least comprises a *dppIV* gene encoding the DPP IV of the invention. This molecule may be in a form of a vector, i.e. a replicative plasmid or an integrative circular or linearized non replicative plasmid. The DNA molecule thus may comprise, operably linked to the *dppIV* gene, regulatory sequences native to the organism from which derives the gene. Said native regulatory sequences may be the promoter, the terminator, and/or a DNA sequence encoding a signal sequence that originally regulated the secretion of the *dppIV* gene, such as the *Aspergillus oryzae* nucleotide sequence coding for a signal peptide from nucleotide 1836 to nucleotide 1966 of SEQ ID NO:1 (without the intron) or functional derivatives thereof due to the degeneracy of the genetic code. In another embodiment, regulatory sequences may be native sequences that regulate a different gene in the said organism of origin or that

regulate a different gene in a foreign organism, for example. A regulatory sequence other than the native regulatory sequence will generally be selected for its high efficiency or desirable characteristic, for example inducibility of a promoter or a sequence encoding a peptide signal which will permit secretion of the protein.

If heterologous expression is preferred, meaning that the genes of the invention are expressed in another organism than the original host (strain, variety, species, genus, family, order, class or division) the regulatory sequences are preferably derived from an organism similar or equal to the expression host. For example, if the expression host is a yeast cell, then the regulatory sequences will be derived from a yeast cell. The promoter suitable for constitutive expression, preferably in a fungal host, may be a promoter from the following genes: glycerolaldehyde-3-phosphate dehydrogenase, phospho-glycerate kinase, triose phosphate isomerase and acetamidase, for example. Promoter suitable for inducible expression, preferably in a fungal host, may be a promoter from the following genes: endoxylanase IIA, glucoamylase A, cellobiosehydrolase, amylase, invertase, alcohol dehydrogenase and amyloglucosidase. The selection of a desirable regulatory sequence operably linked to a sequence of the invention and capable of directing the expression of the said nucleotide sequence is considered to be obvious to one skilled in the art.

The DNA molecule according to the invention may also comprise a selection marker to discriminate host cells into which the recombinant DNA material has been introduced from cells that do not comprise the said recombinant material. Such marker genes are, for example in case fungal expression is preferred, the known *ga-2*, *pyrG*, *pyr4*, *pyrA*, *trpC*, *amdS* or *argB* genes. The DNA molecule may also comprise at least one suitable replication origin. Suitable transformation methods and suitable expression vectors provided with a suitable transcription promoter, suitable transcription termination signals and suitable marker genes for selecting transformed cells are already known in the literature for many organisms including different bacteria, fungal and plant species. In the event fungal expression is required, the expression system described in EP278355 (Novartis) may be thus particularly adapted.

Recombinant koji molds may be obtained by any method enabling a foreign DNA

to be introduced into a cell. Such methods include transformation, electroporation, or any other technique known to those skilled in the art.

5 The invention thus encompasses a recombinant cell comprising the DNA molecule of the invention, the said cell being able to express the DPP IV of the invention or functional derivatives thereof. These cells may be derived from the group of fungal, yeast, bacterial and plant cells. Preferably, yeast cells are of the genera *Saccharomyces*, *Kluyveromyces*, *Hansenula* and *Pichia*, bacterial cells are Gram negative or positive bacteria, i.e. of the genera *Escherichia*, *Bacillus*,
10 *Lactobacillus*, *Lactococcus*, *Streptococcus* and *Staphylococcus*, plant cells are of the vegetable group, and fungal cells are cells that are traditionally used for making a koji, such as *Aspergillus*, *Rhizopus* and/or *Mucor* species, notably *Aspergillus soyae*, *Aspergillus oryzae* (ATCC 20386), *Aspergillus phoenicis* (ATCC 14332), *Aspergillus niger* (ATCC 1004), *Aspergillus awamori* (ATCC
15 14331), *Rhizopus oryzae* (ATCC 4858), *Rhizopus oligosporus* (ATCC 22959), *Rhizopus japonicus* (ATCC 8466), *Rhizopus formosaensis*, *Mucor circinelloides* (ATCC 15242), *Mucor javanicus*, *Penicillium glaucum* and *Penicillium fuscum* (ATCC 10447). Strains referred by an ATCC number are accessible at the American Type Culture Collection, Rockville, Maryland 20852, US. The
20 invention is not limited by such indications which were rather given to enable one skilled in the art to carry out the invention.

Recombinant cells of the invention may comprise the DNA molecule of the invention stably integrated into the chromosome or on a replicative plasmid.
25 Among all recombinant cells of the invention thus created, the present invention has particularly for object the strains *A. oryzae* CNCM I-1887, *A. oryzae* CNCM I-1888 and *Pichia pastoris* CNCM I-1886.

Preferably, functional copies of the *dppIV* gene are integrated at a predefined
30 locus of the chromosomal DNA of the host cell.

Accordingly, in order to operably integrate into the chromosome of prokaryotic cells at least one functional *dppIV* gene which is not fused to any promoter, the DNA molecule of the invention may be integrated by using the process described
35 in EP564966, i.e.,

(1) transforming a host strain organism with a donor plasmid which does not replicate in the host strain, wherein the donor plasmid comprises a vector backbone and a *dppIV* gene of the invention operably integrated, without any promoter, into a part of an operon of the host strain, maintaining the frame and the function of the genomic operon of the host strain; (2) identifying cointegrate transformants in which the complete donor plasmid is integrated into the genomic operon of the host strain; and (3) selecting an integrant transformant from the cointegrate transformants, wherein the genome of the selected integrant transformant does not include the vector backbone of the donor plasmid but does include the *dppIV* gene, which is operably integrated into the conserved genomic operon and which is stably maintained and expressed due to selective pressure on the correct functioning of the essential cistron upon growth in a standard medium.

In a second embodiment, in order to stably integrate into the chromosome of eucaryotic cells only one functional *dppIV* sequence which is fused to a promoter and a terminator which are native to the host organism, DNA molecule of the invention may be integrated by slightly adapting the process of de Ruiter-Jacobs, Y.M.J.T., Broekhuijsen *et al.* (A gene transfer system based on the homologous *pyrG* gene and efficient expression of bacterial genes in *Aspergillus oryzae*. Curr. Genet. 16: 159-163, 1989), i.e.,

(1) preparing a non-replicative DNA fragment by ligating the *dppIV*, which is operably linked to a promoter and terminator that are native to the host organism, downstream a DNA sequence encoding any essential gene, said essential gene being inactivated by at least a mutation and/or a deletion (this essential gene may be a gene involved in uracil biosynthesis, such as the *pyrG* gene in case *A. oryzae* is used, for example); (2) selecting a host organism containing the essential gene which is however inactivated by another mutation(s) or deletion(s); (3) transforming said host organism with the non-replicative DNA fragment; (4) identifying integrate transformants in which the DNA fragment is integrated so as to restore the native function of the essential gene; (5) selecting an integrate transformant in which only one DNA fragment is integrated.

Progeny of an expression host comprising a DNA molecule according to the invention is also included in the present invention. Accordingly, a preferred embodiment of the invention is directed to a cell comprising a recombinant DNA

molecule of the invention in any of the embodiments described above, wherein the said cell is able to integrate the DPP IV into the cell wall or the cell membrane or secrete the enzymes into the periplasmic space or into the culture medium. The secreting route to be followed by the recombinant protein according to the invention will depend on the selected host cell and the composition of the recombinant DNA according to the invention. Most preferably, however, the protein will be secreted into the culture medium. To this end, the cell according to the invention may comprise a recombinant *dppIV* gene further operably linked to a DNA encoding a foreign leader sequence (pre or prepro), for example.

Cells over-expressing the DPP IV of the invention are preferably chosen, especially *Aspergillus* cells capable of providing at least 50 mU, especially at least 100 mU, of DPP IV activity per ml of supernatant when grown in a minimal medium containing 1 % (w/v) of wheat gluten, such as the MMWG medium.

These cells may be obtained by incorporation of the DNA molecule of the present invention in an expression host, said DNA molecule comprising one or more regulatory sequences which serve to increase expression levels of the protein(s) of the invention.

The over-expression can be further achieved by introducing multicopies of the DNA molecule of the invention, for example. Surprisingly, *Aspergillus* cells having integrated multiple recombinant functional *dppIV* genes of the invention may provide a DPP IV activity per ml of supernatant which is more than it should have been compared to the number of integrated copies, probably due to the titration of a negatively acting transcription factor. As an example, the *Aspergillus oryzae* transformant 6 of the following example 1 was deposited under the Budapest Treaty at the CNCM where it receives the deposit number CNCM I-1888.

In addition, it has also been shown that over-expression of the DPP IV may be achieved in *Aspergillus* species naturally providing a prolyl-dipeptidyl-peptidase activity, by integrating multiple copies of the *Aspergillus* native promoter which naturally directs the expression of the gene encoding the prolyl-dipeptidyl-peptidase activity. The promoter region of *Aspergillus oryzae* contained in the nucleotide sequence from nucleotide 1 to nucleotide 1835 of SEQ ID NO:1 is of

particular interest for this purpose. As an example, the *Aspergillus oryzae* transformant B2 of the following example 4 was deposited under the Budapest Treaty at the CNCM where it receives the deposit number CNCM I-1887.

- 5 The invention is also directed to a process for producing the DPP IV of the invention comprising, providing recombinant cells according to the invention in a suitable growth medium under conditions that the cells express the DPP IV, and optionally isolating the said recombinant protein(s) in the form of a concentrate. The selection of the appropriate medium may be based on the choice of expression
10 host and/or based on the regulatory requirements of the DNA recombinant material. Such media are well-known to those skilled in the art.

- After fermentation, the cells can be removed from the fermentation broth by centrifugation or filtration. Depending on whether the host cells have secreted the
15 DPP IV of the invention into the medium or whether the DPP IV are still connected to the host cells in some way either in the cytoplasm, in the periplasmic space or attached to or in the membrane or cell wall, the cells can undergo further treatment to obtain the recombinant protein. In the latter case, where the recombinant enzyme is still connected to the cells, recovery may be accomplished
20 by rupturing the cells for example by high pressure, sonication, enzymatic digestion or simply by cell autolysis followed by subsequent isolation of the desired product. The DPP IV can be separated from the cell mass by various methods, such as ultrafiltration, and then subsequently precipitated with an organic solvent. The isolated DPP IV may be further purified by conventional
25 methods such as precipitation and/or chromatography.

- The present invention also relates to the use of the purified DPP IV or the above mentioned cells to hydrolyse protein containing materials, such as mixtures of a source of proteins and a source of carbohydrates, especially of a mixture of a
30 leguminous plant or of a cooked oleaginous plant and of a cooked or roasted cereal source, for example of a mixture of soya or cooked beans and of cooked or roasted wheat or rice. Compositions containing wheat gluten are particularly adapted for the purpose of the present invention, since considerable amount of glutamine remains sequestered in proline containing peptides when wheat gluten
35 is hydrolysed by traditional koji cultures.

To obtain a satisfactory degree of hydrolysis, the purified DPP IV may suitably be added to the proteinaceous material in a amount of 0.05-15 Unit/ 100 g of protein, in particular 0.1-8 Unit/100g of protein. The incubation may be performed at a pH from between about 4 and about 10, preferably between about 5 and about 9. The
5 incubation may be performed at any convenient temperature at which the enzyme preparation does not become inactivated, i.e. in the range of from about 20°C to about 70°C.

In addition, in the event one may try, after or during hydrolysis with DPPIV, to
10 further liberate as much as possible glutamine linked to proline residues, the present invention provides a method in which the DPP IV of the invention is used in combination with at least an enzyme providing a prolidase activity that is to say an enzyme which has a high level of specificity towards dipeptides of the X-Pro type (Ezespla *et al.*, Ap. Env. Microb., 63, 314-316, 1997; Such kind of enzyme is
15 already available from Sigma: E.C. 3.4.13.9).

In a further aspect, the present invention relates to a food product comprising a protein hydrolysate obtainable by fermentation with at least a microorganism providing a prolyl-dipeptidyl-peptidase activity higher than 50 mU per ml when
20 grown in a minimal medium containing 1 % (w/v) of wheat gluten.

Important food products of the present invention is an ingredient of a mother milk substitute for infants, or a hydrolysed vegetable protein ingredient, i.e. a koji. Indeed, if the DPP IV activity (enzyme or microorganism) is combined with other
25 proteolytic activities (enzymes or microorganisms), i.e. typically if *Pichia pastoris* CNCM I-1886 or *Aspergillus oryzae* CNCM I-1887 or CNCM I-1888 or enzyme purificates thereof are used, high degree of hydrolysis may be obtained leading to a non-bitter flavour and a significantly lower allergenicity than unhydrolysed proteins. The milk substitute may be further formulated in substantially the same
30 way as that indicated in the prior literature for products of this type (cf. EP 96202475.8).

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention, in addition to
35 those described herein, will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended

to fall within the scope of the claims. Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties to the extent necessary for understanding the present invention. DNA manipulation, cloning and transformation of bacteria cells are, except where otherwise stated, carried out according to the textbook of Sambrook *et al.* (Sambrook *et al.*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, U.S.A., 1989). These examples are preceded by a brief description of the plasmids and strains used, and by the composition of various media. The strains *A. oryzae* TK3, *A. oryzae* transformant 6 (example 1), *A. oryzae* transformant B2 (example 4), *Pichia pastoris* containing pKJ115 (example 3) were deposited under the Budapest Treaty, at the Collection Nationale de Culture de Microorganismes (CNCM), 25 rue du docteur Roux, 75724 Paris, France, on June 24, 1997, where they receive respectively the deposit numbers CNCM I-1882, CNCM I-1888, CNCM I-1887 and CNCM I-1886. All restrictions as to the availability of these deposits will be withdrawn upon first publication of this application or another application which claims benefit of priority to this application.

Strains and plasmids

- 20 - *Aspergillus oryzae* 44 and TK3 originate from the Nestlé strain collection. However other wild type *Aspergillus oryzae* strains may also have been used in the context of the following examples.
- *A. oryzae* NF1 derived from TK3 by targeted disruption (uridine auxotrophe).
- *Aspergillus nidulans* 033 (*biA1*, *argA1*) can be obtained through Fungal Genetic Stock Center, Glasgow, and is used as a source of *pyrG* (GenBank accession number M19132) gene. However other wild type *Aspergillus nidulans* strains may also have been used in the context of the following examples.
- 25 - The *Pichia pastoris* (Invitrogen Inc., US)
- Plasmid pMTL21-H4.6 containing the *Aspergillus fumigatus dppIV* gene can be provided by the Institut Pasteur, Paris, France (Beauvais *et al.*, An homolog of the CD26 is secreted by the human pathogenic fungus *Aspergillus fumigatus*, Infect. immun. In press., 1997; GenBank EMBL, accession number: V87950).
- 30 - Plasmid pNFF28 contains the *A. oryzae* TK3 *pyrG* gene (GenBank EBI/UK, accession number: Y13811).
- 35 - Plasmids pMTL20 (Chambers *et al.*, Gene, 68, 139-149, 1988; GenBank EMBL, accession number: M21875), pNEB193 (Biolabs, New England) and

pBluescriptSK⁻ (Stratagene, US) were used in subcloning procedures.

- Plasmid pCL1920b is a derivative of plasmid pCL1920 (Lerner and Inouye, Nucleic Acids Research, 18, 4631, 1990) in which the multiple cloning site was modified to include a *Sma*I site and a *Eco*RI site between the *Bam*HI and *Sal*I sites.

- The *P. pastoris* expression vector pKJ115 was constructed by cloning the expression cassette of pPIC9 (Invitrogen) in pCL1920b. In pKJ115 the expression cassette of pPIC9 is flanked by two *Sma*I sites for linearisation of the DNA, before transformation of *P. pastoris*.

Growth media

- *Aspergillus oryzae* can grow on the minimal medium (MM) prepared according to Pontecorvo *et al.* (Adv. Genet., 5, 141-239, 1953).

15 - *Aspergillus oryzae* NF1 is grown at 35°C on MM containing 10 mM NaNO₃ as a nitrogen source and 10 mM uridine.

- MMWG contains MM plus 1 % (w/v) of wheat gluten (WG) (Sigma),

20 - MMWGH contains MM and 0.1 % (w/v) WG (Sigma) plus 0.1 % (w/v) WG hydrolysate prepared hydrolysing non-vital wheat gluten powder (Roquette, France) with Alcalase 2.4L (Novo Nordisk, Denmark). Hydrolysis is conducted at 20 % (w/w) substrate concentration and an enzyme to substrate ratio (E/S) of 1:50 (by weight of protein) for 6 h at 60°C and constant pH of 7.5 (pH stat). Alcalase is then heat inactivated at 90°C for 10 min. After centrifugation of the hydrolysate, the supernatant is lyophilised to give WGH and stored at room
25 temperature. WGH contains mainly peptides and only minimal amounts of free amino acids. Peptide mass distribution in WGH is from 200 to 10'000 Da, determined by size-exclusion chromatography on a Superdex Peptide column.

30 - *P. pastoris* can grow on RDB (Regeneration Dextrose Base): 1M sorbitol, 1 % (w/v) dextrose, 1.34 % (w/v) yeast nitrogen base (YNB), 4 x 10⁻⁵ % (w/v) biotine, 5 x 10⁻³ % aa (*i.e.* 5 x 10⁻³ % (w/v) of each L-glutamic acid, L-methionine, L-lysine, L-leucine and L-isoleucine.

- MMM (Minimal Methanol Medium): 1.34 % (w/v) YNB, 4 x 10⁻⁵ % (w/v) biotine, 0.5 % (w/v) methanol.

35 - BMGY (Buffered minimal Glycerol-complex Medium): 1 % (w/v) yeast extract, 2 % (w/v) peptone, 10 mM potassium phosphate pH 6.0, 1.34 % (w/v) YNB, 4 x 10⁻⁵ % (w/v) biotine, 1 % (w/v) glycerol.

- BMMY : (Buffered minimal Methanol-complex Medium): 1 % (w/v) yeast extract, 2 % (w/v) peptone, 10 mM potassium phosphate pH 6.0, 1.34 % YNB, 4×10^{-5} % (w/v) biotine, 0.5 (w/v) % methanol.

5 Example 1 Cloning of the *dppIV*

10 - Screening of a genomic library: a genomic DNA library was prepared using the DNA from *A. oryzae* 44 and screened with a DNA fragment containing the *dppIV* gene of *Aspergillus fumigatus* (Beauvais *et al.*, GenBank EMBL, accession number: V87950).

15 For this purpose, the isolation of the genomic DNA was performed according to a modified protocol of the method described by Raeder and Broda (Let. appl. Microbiol., 1, 17-20, 1985). Mycelium was harvested by filtration, immediately
20 frozen in liquid nitrogen and lyophilised. It was then grinded to a fine powder using a mortar and pestle. 200 mg of the powdered mycelium was resuspended in 2.5 ml of extraction buffer (200 mM Tris-HCl pH 8.5 150 mM NaCl, 25 mM EDTA, 0.5 % SDS) and the solution was extracted with 1.75 ml extraction buffer-equilibrated phenol and 0.75 ml of chloroform/isoamylalcohol (24:1, v/v). The
25 mixture was centrifuged (20 min, 3000 g). The aqueous phase was retrieved and incubated with 125 µl of RNase A (Boehringer) solution (10 mg/ml) for 10 min at 37°C. 1.25 ml of 2-propanol (Merck) were then added. The pellet was washed with 70 % ethanol and finally resuspended in 500 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). 500 µl of 2 x QBT (1.5 M NaCl, 100 mM MOPS, 30
30 % ethanol, pH 7.0) were added to the sample which was then applied to a "Genomic-tip" (Qiagen), rinsed and eluted as recommended by the supplier.

30 The genomic DNA was then partially digested with *Sau3A*, and DNA fragments of 12-20 kb were isolated from low melting agarose (Biorad). These fragments were inserted into bacteriophages using the λ EMBL3 *Bam*HI arm cloning system (Promega, US):

35 40000 recombinant plaques of the *A. oryzae* 44 genomic library in λ EMBL3 were immobilised on nylon membranes (Genescreen, Dupont). These filters were probed, with the 32 P-labelled 2.3 kb *dppIV* insert of pMTL21-H4.6 amplified by PCR in a 5 x SSC solution containing 20 % formamide, 1 % sodium dodecyl

sulfate (SDS), and 10 % dextran sulfate at 42°C for 20 h. Labelling of DNA was performed using a random-primed DNA labelling kit (Boehringer) and ($\alpha^{32}\text{P}$)-dATP. The membranes were exposed to X-ray film after two 20 min washes in 3 x SSC-1 % SDS at 40°C.

5

Ten positive clones were isolated and purified. Restriction enzyme analysis of purified bacteriophage DNA revealed that the clones carried similar but not identical DNA fragments. By Southern analysis, the *dppIV* gene was assigned to an *ApaI-EcoRV* 4.8 kb fragment which was subcloned into pBluescriptSK⁺, creating the plasmid pNFF125.

10

- Checking of functionalities: plasmid pNFF125 was introduced into *A. oryzae* NF1 by cotransformation with plasmid pNFF28, carrying the *pyrG* gene for selection of transformants.

15

For this purpose, *A. oryzae* NF1 was grown overnight in MM with 50 mM glucose, 5 mM glutamine and 10 mM uridine. The mycelium was harvested by sterile over cheese cloth filtration, washed once with sterile double distilled water and once with K0.8MC (20 mM MES-HCl pH 5.8, 0.8 M KCl, 50 mM CaCl_2). 2 g of mycelium were resuspended in 20 ml of a filter sterilised 5 mg/ml solution of Novozyme 234 in K0.8MC. The mycelium suspension was incubated at 30°C for 2 hours with gentle agitation (120 rpm). The protoplasts were liberated from the mycelium by gentle resuspension with a pipette, washed twice with 20 ml of S1.0TC (10 mM Tris-HCl pH 7.5, 1 M Sorbitol, 50 mM CaCl_2) and were resuspended at a final concentration of 10^8 /ml in S1.0TC. 20 ml of DNA was mixed with 200 ml of protoplasts and 50 ml of 25 % PEG 6000 (BDH) in 10 mM Tris-HCl pH 7.5, 50 mM CaCl_2 and incubated for 20 min on ice. To this mixture, 2 ml of 25 % PEG 6000 in 10 mM Tris-HCl pH 7.5, 50 mM CaCl_2 were added, gently mixed and incubated for 5 min at room temperature. 4 ml of S1.0TC was added and 1.0 ml aliquots were mixed with 5 ml of 2 % low melting point agarose (Sigma) SMM (MM plus 50 mM glucose and 5 mM glutamine, osmotically stabilised with 1.0 M sucrose) and plated onto SMM agar (Difco).

20

25

30

Ninety-five *pyrG*⁺ transformants were screened for DPP IV activity after incubation (2 days, 30°C) on MMWGH. For this purpose, spores of transformants were resuspended in SP2 buffer (20 mM KH_2PO_4 adjusted to pH 2.0 with HCl and

35

0.9 % NaCl) in microtiter plates and replica plated onto Petri dishes containing MMWGH covered by a Whatman filter (Chr1). The plates were incubated for 2 days at 30°C. DPP IV activity was detected on the filter according to Lojda (Histochemistry, 54, 299-309, 1977) and Aratake et al. (Am. J. Clin. Pathol., 96, 306-310, 1991). Filters were reacted with a solution of 3 mg glycyl proline 4-β naphthylamide (Bachem) in 0.25 ml N, N-dimethylformamide (Merck) and 5 mg o-dianisidine, tetrazotized (Sigma) in 4.6 ml 0.1 M sodium phosphate buffer pH 7.2 for 10 min at room temperature. Endoproteolytic enzyme activity was also measured with resorufin-labeled casein according to Boehringer method description supplied with the substrate (Resorufin-labeled casein, Cat.No. 1080733). Leucine aminopeptidase and dipeptidyl peptidase IV activities were determined by UV spectrometry with synthetic substrates Leu-pNa and Ala-Pro-pNa (Bachem, Switzerland), respectively, according to Sarath *et al.* (Protease assay methods in Proteolytic enzymes: a practical approach, IRL Press, Oxford, 1989). 10 mM substrate stock solution in dimethylsulfoxide (DMSO) was diluted with 100 mM sodium phosphate buffer, pH 7.0, to a final concentration of 0.5 mM. 20-100 µl culture medium supernatant was added and reaction proceeded for up to 60 min at 37°C. A control with blank substrate and blank supernatant was done in parallel. The release of the chromophoric group 4-nitroaniline (ϵ : 10'500 M⁻¹cm⁻¹) was measured at 400 nm and activities were expressed as mU/ml (nmol/min/ml).

Results show that sixteen transformants exhibited a clearly increased staining compared to the wild type. Seven transformants numbered 1 to 7 were selected because of their high DPP IV activity. Southern blots of them confirmed that the increase in the activity was due to the integration of multiple copies of the 4.8 kb *Apal-EcoRV* fragment in the genome of the transformants. From densitometric scans of these Southern blots, it was estimated that in transformant 1, at least 4 additional copies had been functionally integrated into the genomic DNA, while, in transformant 6, they were at least 9 additional copies.

To quantify the increase of DPP IV activity in the transformants 1 and 6, these were grown in parallel with control *A. oryzae* NF1 *pyrG*⁺, for 7 days at 30°C without shaking in 100 ml liquid MMWG. Analyses of the supernatants are shown in Table 1. Transformants 1 and 6 showed a DPP IV activity of at least 8 and 17 times more, respectively, than *A. oryzae* NF1 *pyrG*⁺ transformant, while their

leucine-aminopeptidase (LAP) and endopeptidase (ENDO) activities remain unchanged. These data strongly suggested that pNFF125 contained a functional *dppIV* gene. In addition, when a functional gene was introduced, the DPP IV activity increased more than it should have been compared to the number of integrated copies. The difference might also come from the titration of a negatively acting transcription factor (repressor).

Table 1

	DPP IV [mU/ml]	LAP [mU/ml]	ENDO [mU/ml]
NF1 <i>pyrG</i> ⁺	8.7	1.6	2.9
Transformant 1	73.9	1.7	3.3
Transformant 6	160.6	1.9	3.1

- Characterisation of the DPP IV: culture broth from prolyl dipeptidyl peptidase overproducing transformant 6 and the control *A. oryzae* NF1 *pyrG*⁺ were analysed by SDS-PAGE. No single band in the prolyl dipeptidyl peptidase-overproducing strain stained more intensely than the *A. oryzae* NF1 *pyrG*⁺ control. However, a broad smear was visible in the region around 95 kDa of the prolyl dipeptidyl peptidase-overproducing strain, but not in the *A. oryzae* NF1 *pyrG*⁺ control. This aberrant electrophoretic behaviour might be caused by glycosylation of the enzyme. Therefore, culture broths were treated with N-glycosidase F and reanalysed. In the deglycosylated samples a band of 85 to 90 kDa appeared in the control NF1 *pyrG*⁺ and in the prolyl dipeptidyl peptidase overproducing transformant. A sample of the N-glycosidase F treated culture medium of transformant 6, corresponding to 100 mU prolyl dipeptidyl peptidase activity, was loaded onto a preparative gel and blotted onto an Immobilon P^{SO} membrane. The putative prolyl dipeptidyl peptidase band was excised and analysed by automated Edman degradation. The N-terminal sequence of the mature protein was determined to be Leu-Asp-Val-Pro-Arg-....

- Sequencing of the *ApaI-EcoRV* fragment: the 4.8 kb fragment from pNFF125 was sequenced on both strands. The nucleotide sequence of the *dppIV* gene was determined, on a Licor model 4000 automatic sequencer. IRD41 labelled primer having the nucleotide sequence SEQ ID NO:3 was used for sequencing both strands of partially overlapping subclones by the dideoxynucleotide method of Sanger *et al.* (Proc. Natl. Acad. Sci. USA, 74, 5463-5467, 1977). The DNA

sequence analysis was performed by using the GCG Computer programs (Devereux *et al.*, Nucl. Acids Res., 12, 387-395, 1987).

The position of transcription start sites were mapped by primer extension. Additionally the position of exons and intron were determined by RT-PCR. For this purpose, total RNA was isolated from the *A. oryzae* TK3 mycelia cultured overnight on MMWGH, using the "RNeasy Total RNA Purification kit" (Qiagen). Reverse transcriptase PCR (RT-PCR) was performed using the "1st strand cDNA synthesis kit for RT-PCR" (Boehringer). 10 µg of total RNA, 1 x reaction buffer (10 mM Tris, 50 mM KCl pH 8.3), 5 mM MgCl₂, 1 mM deoxynucleotide mix, 1.6 µg oligo-p(dT)₁₅ primer, 50 units RNase inhibitor, 10 units AMV Reverse transcriptase were mixed and incubated 25°C 10 min, 42°C 60 min, 75°C 5 min and 4°C 5 min. 1 µl, 2 µl and 3 µl of the obtained cDNA, 2 mM of oligonucleotides and 250 mM dNTPs (Boehringer) were dissolved in 50 µl of 1 x PCR buffer (20 mM Tris-HCl pH 8.55, 16 mM (NH₄)₂SO₄, 2.5 mM MgCl₂, 150 mg/ml BSA). To each reaction 1.5 unit of Taq-polymerase (Biotaq) were added as well as one drop of Nujol mineral oil (Perkin Elmer). The targeted region of the *dppIV* gene was amplified, using a Stratagene Robo Cyclor gradient 40, with the primer pair SEQ ID NO: 4 and SEQ ID NO:5. The reaction mixtures were subjected to 2 cycles of 1 min 98°C, 2 min 56°C and 2 min 72°C, followed by 27 cycles of 1 min 94°C, 1 min 56°C and 2 min 72°C and 1 cycle of 1 min 94°C, 1 min 56°C and 10 min 72°C. The gel purified PCR products were recovered with Qiaex II (Qiagen) and directly ligated into the pGEM-T vector (Promega) according to the instructions of the manufacturer, to generate plasmid pNFF137.

Results show that the open reading frame (ORF) is split by a 83 bp intron into 2 exons. Furthermore, the 16 aa long N-terminal secretory signal sequence was identified by homology with the *A. fumigatus* sequence which corresponds well to the signal sequence rule described by Von Heijne (Nucleic Acids Res., 14, 4683-4690, 1986). The *dppIV* gene has the nucleotide sequence SEQ ID NO:1, and encodes a mature protein of 755 aa with a deduced molecular weight of 85.4 kDa (see SEQ ID NO:2). The signal sequence of *dppIV* runs from position 1835 (ATG) to 1966 and includes the intron. The mature protein starts at position 1967 with the amino acid sequence LeuAspValProArg as confirmed by Edman degradation. The exon I starts at position 1836 and ends at position 1841; intron starts at position 1842 and ends at position 1924; exon II starts at position 1925

and ends at position 4231.

Example 2 Disruption of the *dppIV* gene

- 5 In order to determine if the cloned *dppIV* gene was exclusively responsible for the DPP IV activity observed onto MMWGH, it was disrupted.

As heterologous selection marker, to prevent targeting of the disrupting construct to the *pyrG* locus, the *A. nidulans pyrG* gene was amplified from *A. nidulans* 033.
10 To do so, the sequences between position 500 and 2342 of the *pyrG* gene (Oakley, et al., Gene, 61, 385-399, 1987) were amplified by PCR. 200 ng *A. nidulans* 033 genomic DNA, 2 mM of oligonucleotides and 250 mM dNTPs (Boehringer) were dissolved in 50 ml of 1 x PCR buffer (20 mM Tris-HCl pH 8.55, 16 mM (NH₄)₂SO₄, 2.5 mM MgCl₂, 150 mg/ml BSA). To each reaction 1.5 unit of Taq-polymerase (Biotaq) were added as well as one drop of Nujol mineral oil (Perkin Elmer). The targeted region was amplified, using a Stratagene Robo Cycler gradient 40, with the primer pairs SEQ ID NO:6 and SEQ ID NO: 7. The reaction mixtures were subjected to 30 cycles of 1 min 95°C, 1 min 52°C and 3 min 72°C. The gel purified 1.8 kb PCR product was recovered with Qiaex II (Qiagen) and
15 cloned into pGEM-T (Promega), according to the instructions of the manufacturer, to give pNFF39.
20

In parallel, a mutant allele of *dppIV* was generated from pNFF 125 by replacing the internal 1.5 kb *NcoI* fragment with the 1.8 kb *NcoI* fragment from pNFF39,
25 creating pNFF129.

Apal-EcoRV digested pNFF129 was introduced into *A. oryzae* NF1 and the transformants were grown on MM. Among 95 tested on MMWGH, eighteen transformants did not exhibit DPP IV activity. Six DPP IV negative transformants
30 were selected and numbered from 8 to 13, and four transformants which still exhibited DPP IV activity were numbered from 14 to 17. A Southern blot of *NcoI* digested genomic DNA from these ten transformants was probed with the *dppIV* PCR fragment (see example 2). In transformants which did not exhibit DPP IV activity, the 1.5 kb *NcoI* fragment is absent, which proves that the wild type gene has been replaced by the disruption construct. In transformants which retain DPP
35 IV activity, the 1.5 kb fragment is still present, and hybridising fragments with

other molecular weights show that the disruption construct has integrated at another site in the genome.

To quantify DPP IV activity, transformants 10, 11 and 15 as well as *A. oryzae* NF1 *pyrG*⁺ transformant were grown for 7 days at 30°C on liquid MMWG. Enzymatic analyses of the supernatant (table 2) showed that transformants 10 and 11 had residual proline dipeptidyl-peptidase activity, probably due to some non specific enzymes. By contrast, transformant 15 had a higher DPP IV activity (at least 4 times more) compared to the wild type. Inspection of the original screen for DPP. IV disruption mutant revealed additional clones with higher activity compared to the wild type. Since the disruption construct did not contain a functional gene, the increase of the activity might have been due to titration of a repressor.

Table 2

	DPP IV [mU/ml]	LAP [mU/ml]	ENDO [mU/ml]
NF1 <i>pyrG</i> ⁺	8.7	1.6	2.9
Transformant 10	0.4	5.8	3.1
Transformant 11	0.1	6.5	4.5
Transformant 15	39.6	5.7	2.7

Example 3 Expression of *A. oryzae* DPP IV in *P. pastoris*

- Transformation of *P. pastoris*: plasmid pNFF125 was used as template for multiplying the *dppIV* gene by PCR. To do so, 200 ng of pNFF125 DNA, 164 pmol of oligonucleotides, 120 mM dNTP's were dissolved in 50 ml PCR buffer (20 mM Tris-HCl pH 8.8, 2 mM MgSO₄, 10 mM (NH₄)₂SO₄, 0.1 % Triton X-100, 100 mg/ml nuclease free BSA). A drop of dynawax (Dynazyme) was added. To each reaction 2.5 unit of cloned Pfu DNA polymerase (Stratagene) was added in 50 ml of 1 x PCR buffer. The *A. oryzae dppIV* gene was amplified with the primer pair SEQ ID NO:8 and SEQ ID NO:9 (these primers covered N- and C- terminal mature protein coding region). The reaction mixtures were subjected to thirty cycles of 1 min 95°C, 1 min 44°C and 3 min 72°C using Perkin Elmer DNA Thermal Cycler.

The PCR product was digested by *EcoRV* and *NotI* and cloned into the *SnaBI*,

NotI digested pKJ115, generating the plasmid pNFF134. *P. pastoris* sphaeroplasts were transformed with 10 µg of pNFF134 linearised by *EcoRI* as described in the Manual Version 2.0. of the *Pichia* Expression Kit (Invitrogen).

- 5 The *P. pastoris* expression cassette pKJ115 can insert into the *P. pastoris* genome via homologous recombination at the alcohol oxidase (AOX1) site and carry, in addition to the cloned coding sequence of interest, the *his4* gene for selection. Transformants were first selected on histidine-deficient media (RDB) and then
10 screened for insertion of the construct at the *aox1* site on minimal methanol plates (MMM). Transformants that were unable to grow on media containing only methanol as a carbon source (BMMY) were assumed to contain the construct in the correct yeast genomic location by integration events at the *aox1* locus displacing of the *aox1* coding region. The selected transformants were grown to near saturation (OD 20 at 600 nm) at 30°C in 10 ml of glycerol-based yeast media
15 (BMYG). Cells were harvested and resuspended in 2 ml BMYG and incubated for 2 days. After two days of incubation, the supernatant was harvested and 10 ml was analysed by SDS-PAGE according to the method of Laemmli (1970) with a separation gel of 7.5 % (w/v) polyacrylamide to identify successfully expressing clones. In parallel, the supernatant was checked for activity.

20

- Results show that the obtained concentration of DPP IV was 100 µg/ml. The activity measured in the supernatant was of about 1385 mU/ml. Among all the transformants, one was deposited under the Budapest Treaty at the Collection Nationale de Cultures de Microorganismes (CNCM), 25 rue du Docteur Roux,
25 75724 Paris, France, on June , where it receives the deposit number CNCM I-3.

- Peptide profiling by size exclusion chromatography (SEC): the efficiency of DPP IV towards peptides in WG hydrolysates was tested. Enzymes in the supernatant of *dppIV* disruptant 11 thus were heat inactivated at 95°C for 10 min.
30 140 mU of purified DPP IV produced by *P. pastoris* CNCM I-3 were added to 500 µl of supernatant and incubated at 45°C up to 24 h. A control experiment without DPP IV addition was performed in parallel. Aliquots were taken at 2 h intervals, acidified with 10 % TFA, centrifuged and analysed by SEC on a Superdex Peptide HR 10/30 column (Pharmacia Biotech, Sweden). Separation is
35 based on molecule size of amino acids and peptides (range: 100-7'000 Da). Chromatography was performed under isocratic conditions with 0.1 % TFA, 20 %

acetonitrile in water at a flow rate of 0.5 ml/min. Detection of amino acid and peptide peaks was at 215 nm. Peptide and amino acid standards were used to calibrate the chromatographic system (data not shown).

- 5 Results show that an initial increase of small peptides (200-500 Da) can be detected already after 2 h incubation. Extended incubation (up to 24 h) releases more dipeptides. No changes are detected in the control sample at 2 h and 24 h incubation time. Therefore, it is clear that DPP IV activity liberates dipeptides from wheat gluten hydrolysates confirming the efficiency of this enzyme in
10 peptide degradation

Example 4 Transformation with the native promoter of *dppIV*

- The plasmid pNFF126 containing the fragment of 2094 bp *ApaI-BamHI* encompassing the promotor region and the start of the DPP IV gene (see SEQ ID
15 NO:1) was introduced into *A. oryzae* NF1, using *pyrG* gene as selection marker. The *A. oryzae* NF1 *pyrG*⁺ transformants were screened by staining for their prolyl-dipeptidyl-peptidase activity. Two transformants (B2, C7) showed a more intensive stain than the other ones. They were therefore cultured onto liquid
20 MMWG for 7 days, 30 °C, without shaking, in parallel with three other randomly picked transformants and the control *A. oryzae* NF1 transformed with only *pyrG*.

- The prolyl-dipeptidyl-peptidase activity was analysed from the culture broths. Results show that transformants B2 and C7 respectively showed a fourfold and
25 twofold increase of the prolyl dipeptidyl peptidase activity compared to the control, whereas all the other ones do not exhibit any increase of this activity. In the disruption experiment (see example 2), also a maximum of fourfold increase of the prolyl-dipeptidyl-peptidase activity was noticed (transformant 15). This increase can be due to a repressor titrated by the multicopies of the promotor
30 region integrated heterologously in the genome of *A. oryzae* NF1 or by a positive acting factor encoded by the 2094 bp *ApaI-BamHI* fragment.

Example 5 Functional derivatives of the DPPIV

- 35 Functional derivatives of the DPP IV (SEQ ID NO:2) are prepared according to a method adapted from the method described by Adams et al. (EP402450;

Genencor). Briefly, the expression cassette pKJ115 containing the DPP IV was subjected to an *in-vitro* chemical mutagenesis by hydroxylamine. According to example 3, the mutagenised DNA was then used to transform *P. pastoris*. Functional derivatives of the DPP IV, presenting a deletion, addition and/or a substitution of some amino acids, were finally detected according to their peptide profile obtained by hydrolysing wheat gluten with purified DPP IV derivatives (see example 3).

Examples 6

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For preparing a fermented soya sauce, a koji is prepared by mixing an *Aspergillus oryzae* CNCM I-1 koji culture with a mixture of cooked soya and roasted wheat, the koji is then hydrolysed in aqueous suspension for 3 to 8 hours at 45°C to 60°C with the enzymes produced during fermentation of the *Aspergillus oryzae* CNCM I-1 culture, a moromi is further prepared by adding suitable amount of sodium chloride to the hydrolysed koji suspension, the moromi is left to ferment and is then pressed and the liquor obtained is pasteurized and clarified.

Examples 7

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For producing a flavouring agent, a aqueous suspension of a mixture of cooked soya and roasted wheat is prepared, the proteins are solubilized by hydrolysis of the suspension with a protease at pH6.0 to 11.0, the suspension is heat-treated at pH 4.6 to 6.5, and the suspension is ripened with enzymes of a koji culture fermented by *Aspergillus oryzae* CNCM I-2.

25

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: SOCIETE DES PRODUITS NESTLE
(B) STREET: AV. NESTLE 55
(C) CITY: VEVEY
(D) STATE: VAUD
(E) COUNTRY: SWITZERLAND
(F) POSTAL CODE (ZIP): CH-1550

(ii) TITLE OF INVENTION: CLONING OF THE PROLYL-DIPEPTIDYL-PEPTIDASE
OF ASPERGILLUS ORYZAE

(iii) NUMBER OF SEQUENCES: 9

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5496 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: exon
(B) LOCATION: 1836..1841

(ix) FEATURE:

- (A) NAME/KEY: exon
(B) LOCATION: 1925..4231

(ix) FEATURE:

- (A) NAME/KEY: intron
(B) LOCATION: 1842..1924

(ix) FEATURE:

- (A) NAME/KEY: sig_peptide
(B) LOCATION: 1836..1841

(ix) FEATURE:

- (A) NAME/KEY: sig_peptide
(B) LOCATION: 1925..1967

(ix) FEATURE:

- (A) NAME/KEY: promoter
(B) LOCATION: 1..1835

(ix) FEATURE:

- (A) NAME/KEY: terminator
(B) LOCATION: 4232..4771

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GGGCCCTGAG TTAAACGGTG CTGGGTGTGT TATTACGCAT CATACTCTTC ACCCGCCTTG 60
CAGTAGTTCC GTTCTATTGT CAATAGCTGC TGTCGCAATA TTCTGTCTTT TGCCAATAAG 120
GTGACCAGGA GGGGTCTTTC CAGGATAGAT AGATGGCGAC ATTTATCTCG TCGCGGCGGT 180
GATTGTCTGT TTGATTGATG ATGATCTCTG AAACATGTTG AATCTGGGGT ACGTAACTTG 240
GGGTGATCAA TTGACATCCA CTTAGATATG GTACAGCAA GTATACCTCC TGGATTCTGT 300

	GAACAAGAAT ATAAAAATAAG CCTCGCGACC GGGAGTCTTG TCCCTCAAAT CATCACAATC	360
	CCATCGAACA TCCGCATCTA ATTTCTCTCAC TCATCCTTCT ATCCACCGCC AAAATGAAGG	420
	CCGCTACCCCT CCTCTCTCTT CTGAGCGTTA CCGGACTCGT CGCCGCTGCT CCAGCTGGCA	480
	ACGGTACGTA TCCTGAACGA CAATGTAAGA CGCTTGACTG ATGATTAGTA GGCCAGCTG	540
10	GTGGAATCAT CGACCGCGAT CTTCCCGTCC CTGTCCCTGG ACTCCCTACC AAGGGTCTCC	600
	CTATTGTTGA CGGATTGACT GGCGGCAATA AGGGTGGCGA GAAGCCTGGA AGCAAGGTTA	660
	CTCCTCGTGA AGACCCTACC GGCAGCGCCC CTGATGGCAA GGGCAATGAT GGCCCCGACG	720
	GTGATCTTAC CGGACGTCCC GGTCAAGGGG GTCTTGACAA CCCTTTCGAT CTCCCTACTC	780
	CAGAGCTTCC TCCCCTCAAG CTTCTTGCGG GACTTGACGG TGGCAAGGGC GGTCTCGGCC	840
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(2) INFORMATION FOR SEO ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 771 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

(ix) FEATURE:

(A) NAME/KEY: Peptide

(B) LOCATION: 1..16

(D) OTHER INFORMATION: /label= signal-peptide

(ix) FEATURE:

(A) NAME/KEY: Protein

(B) LOCATION: 17..771

(D) OTHER INFORMATION: /label = secreted-enzyme

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/note = "enzyme providing a  
prolyl-peptidyl-peptidase  
activity"
```

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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[illegible]

Leu Asp Val Pro Arg Lys Pro His Ala Pro Thr Gly Glu Gly Ser Lys
1 5 10 15

Arg Leu Thr Phe Asn Glu Thr Val Val Lys Gln Ala Ile Thr Pro Thr

	20	25	30
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Ser Arg Ser Val Gln Trp Leu Ser Gly Ala Glu Asp Gly Ser Leu Arg
35 40 45

Val Arg Gly Gly Arg Arg Gln Ser His His Arg Glu His Arg His Gln

Val	Mg	Cl₁	Cl₂	Mg	Mg	Si₁	Se₁	NiS	NiS	Arg	Sid	NiS	Arg	NiS	Si₁
50						55					60				

Arg Val Thr His Ala His Pro Ala Asp Lys Ile Pro Thr Gly Lys Glu

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				115						120					125			
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				130				135						140				
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 Gly Gly Val Phe Leu Tyr Lys Gln Leu Ala Arg Lys Leu Tyr Gln Glu
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 755

- 20 (2) INFORMATION FOR SEQ ID NO: 3:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 18 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: DNA (genomic)
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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18

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 (A) LENGTH: 18 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 40 (ii) MOLECULE TYPE: DNA (genomic)
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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18

- (2) INFORMATION FOR SEQ ID NO: 5:
 (i) SEQUENCE CHARACTERISTICS:
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 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 50 (ii) MOLECULE TYPE: DNA (genomic)
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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18

60

- (2) INFORMATION FOR SEQ ID NO: 6:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 23 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: DNA (genomic)
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23

- (2) INFORMATION FOR SEQ ID NO: 7:
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 (A) LENGTH: 24 base pairs
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 (D) TOPOLOGY: linear
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24

- (2) INFORMATION FOR SEQ ID NO: 8:
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 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: DNA (genomic)
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

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TGGTCGATAT CCTGGATGTG CCTCGGAAAC CA

32

40

- (2) INFORMATION FOR SEQ ID NO: 9:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
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 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
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 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

50

TTGCGGCCGC TACTCCTCCA AGTCCTTCTT

30

Claims

1. A recombinant prolyl-dipeptidyl-peptidase (DPP IV) from *Aspergillus oryzae* comprising the amino-acid sequence from amino acid 1 to amino acid 755 of SEQ
5 ID NO:2 or functional derivatives thereof.
2. A recombinant prolyl-dipeptidyl-peptidase according to claim 1 which is fused to a leader peptide.
- 10 3. A recombinant prolyl-dipeptidyl-peptidase according to claim 2 which is fused to the leader peptide of *Aspergillus oryzae* having the amino-acid sequence from amino acid -16 to amino acid -1 of SEQ ID NO:2 or functional derivatives thereof.
4. A leader peptide of *Aspergillus oryzae* having the amino-acid sequence from
15 amino acid -16 to amino acid -1 of SEQ ID NO:2 or functional derivatives thereof.
5. A DNA molecule which comprises a *dppIV* gene encoding the enzyme according to claim 1.
- 20 6. A DNA molecule according to claim 5, which is a vector comprising the *dppIV* gene.
7. A DNA molecule according to claim 5, wherein the *dppIV* gene is operably linked to at least one regulatory sequence able to direct the expression of the gene.
25
8. A DNA molecule according to claim 7, wherein the regulatory sequence is derived from another organism than the one from which the *dppIV* gene is derived.
- 30 9. A DNA molecule according to claim 5, wherein the *dppIV* gene comprises the coding parts of the nucleotide sequence SEQ ID NO:1 or functional derivatives

thereof due to the degeneracy of the genetic code.

10. A cell which expresses the enzyme according to claims 1-4 by recombinant technology.

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11. A cell according to claim 10, which is *Pichia pastoris* CNCM I-1886.

12. A cell according to claim 10 which is able to over-express the enzyme.

10 13. A cell according to claim 12, which is an *Aspergillus oryzae* capable of providing at least 50 mU of prolyl-dipeptidyl-peptidase activity per ml of supernatant when grown in a minimal medium containing 1% (w/v) of wheat gluten.

15 14. An *Aspergillus oryzae* according to claim 12, wherein it has integrated multiple recombinant functional *dppIV* genes according to claims 5 to 9.

15. An *Aspergillus oryzae* according to claim 14 which is the *Aspergillus oryzae* CNCM I-1888.

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16. An *Aspergillus* naturally providing a prolyl-dipeptidyl-peptidase activity which has integrated multiple copies of the *Aspergillus* native promoter which naturally directs the expression of the gene encoding the prolyl-dipeptidyl-peptidase activity.

25

17. An *Aspergillus* according to claim 16, which has integrated multiple copies of the promoter contained in the nucleotide sequence SEQ ID NO:1.

18. An *Aspergillus* according to claim 17, which has integrated multiple copies of
30 the promoter having the coding nucleotide sequence from nucleotide 1836 to nucleotide 1966 of SEQ ID NO:1.

19. A *Aspergillus oryzae* according to claim 18, which is the *Aspergillus oryzae* CNCM I-1887.

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20. An *Aspergillus* naturally providing a prolyl-dipeptidyl-peptidase activity

which is manipulated genetically so that the *dppIV* gene is inactivated.

21. A method for producing the enzyme according to claim 1, comprising cultivating recombinant cells according to claims 10-19 in a suitable growth medium under conditions that the cells express the enzyme, and optionally isolating the enzyme in the form of a concentrate.
22. Use of the protein according to claim 1 or the cells according to claims 10-19 to hydrolyse protein containing materials.
23. Use of an enzyme and/or a microorganism providing a prolyl-dipeptidyl-peptidase activity, in combination with at least an enzyme providing a prolidase activity, to hydrolyse protein containing materials.
24. A food product comprising a protein hydrolysate obtainable by fermentation of protein containing materials with at least a microorganism providing a prolyl-dipeptidyl-peptidase activity higher than 50 mU per ml when grown in a minimal medium containing 1 % (w/v) of wheat gluten.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 98/02799

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/57 C12N9/48 C12N1/15 C12N1/19 C12P21/06
A23J3/30

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A23J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TACHI H ET AL: "AN X PROLYL DIPEPTIDYL-AMINOPEPTIDASE FROM ASPERGILLUS -ORYZAE." PHYTOCHEMISTRY (OXF) 31 (11). 1992. 3707-3709. CODEN: PYTCAS ISSN: 0031-9422, XP002047735	1-22
Y	see the whole document	23,24
A	BEAUVAIS A ET AL: "Biochemical and antigenic characterization of a new dipeptidyl-peptidase isolated from Aspergillus fumigatus." JOURNAL OF BIOLOGICAL CHEMISTRY 272 (10). 1997. 6238-6244. ISSN: 0021-9258, XP002047736	1-22
Y	see the whole document	
Y	EP 0 417 481 A (NESTLE SA) 20 March 1991	23,24
	see the whole document	
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

Date of the actual completion of the international search

7 September 1998

Date of mailing of the international search report

21/09/1998

Name and mailing address of the ISA

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 98/02799

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>DATABASE WPI Section Ch, Week 9527 Derwent Publications Ltd., London, GB; Class D13, AN 95-202831 XP002047737 & JP 07 115 969 A (ASAHI KASEI KOGYO KK) , 9 May 1995 see abstract</p> <p style="text-align: center;">---</p>	23,24
X	<p>EP 0 754 752 A (AJINOMOTO KK) 22 January 1997</p>	23,24
Y	<p>see the whole document * especially page 9 lines 37-38 *</p> <p style="text-align: center;">---</p>	23,24
X	<p>HEYMANN E ET AL: "COMPLEMENTARY ACTION OF DIPEPTIDYL PEPTIDASE IV AND AMINOPEPTIDASE M IN THE DIGESTION OF BETA CASEIN." J DAIRY RES 53 (2). 1986. 229-236. CODEN: JDRSAN ISSN: 0022-0299, XP002052650</p>	23
Y	<p>see page 232, last paragraph; table 2</p> <p style="text-align: center;">---</p>	23
Y	<p>DATABASE WPI Section Ch, Week 9711 Derwent Publications Ltd., London, GB; Class B04, AN 97-112833 XP002051875 & JP 09 000 249 A (AJINOMOTO KK) , 7 January 1997 see abstract</p> <p style="text-align: center;">---</p>	23
X	<p>CHEMICAL ABSTRACTS, vol. 124, no. 21, 20 May 1996 Columbus, Ohio, US; abstract no. 287456, TACHI, HIROSHI: "X-prolyl- dipeptidyl -aminopeptidase activity during soy sauce fermentation" XP002051874 see abstract & NIPPON JOZO KYOKAISHI (1996), 91(2), 138-40 CODEN: NJKYES;ISSN: 0914-7314, 1996,</p> <p style="text-align: center;">---</p>	24
P,X	<p>BEAUVAIS A ET AL: "Dipeptidyl-peptidase IV secreted by Aspergillus fumigatus, a fungus pathogenic to humans" INFECTION AND IMMUNITY, vol. 65, no. 8, August 1997, pages 3042-3047, XP002076492 WASHINGTON US see the whole document</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	1-22

INTERNATIONAL SEARCH REPORT

Inter: 1st Application No

PCT/EP 98/02799

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>EMBL/GENBANK DATABASES, Accession no AJ002369, Sequence reference A00PPIV 2 March 1998 DOUMAS A ET AL: "Characterization of the prolyl dipeptidyl peptidase-encoding gene (dppIV) from the koji mould <i>Aspergillus oryzae</i>" XP002076626 see the whole document -----</p>	1-22

INTERNATIONAL SEARCH REPORT

I. International application No.

PCT/EP 98/02799

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/SA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims : 1-22 completely, 23 24 partially

DPP IV from *Aspergillus oryzae*, DNA coding for this enzyme and its use plus product thereof.

2. Claim : 23 partially

The use of enzymes or microorganism providing a prolyl-dipeptidyl-peptidase activity other than DPP IV from *A. oryzae* in combination with a prolidase

3. Claim : 24 partially

A food product obtainable by fermentation of protein containing material with a microorganism providing a prolyl-dipeptidyl-peptidase higher than 50mU per ml

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 98/02799

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